SOME PROPERTIES OF CYANOETHYLATED TROPOCOLLAGEN

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Received on December 12, 1968

The reaction of acrylonitrile with tropocollagen was studied under different conditions. The maximum amount of cyanoethyl groups that could be introduced was 0·4 m. moles per g. of collagen and thus showed that the addition of acrylonitrile primarily takes place at the amino groups. Reconstituted cyanoethylated collagen showed the ability to redissolve on cooling and thus resembled lathyritic collagen. The denaturation temperature, viscosity, dissolution temperature and aldehyde content of collagen are lowered by cyanoethylation. The lower stability of cyanoethylated collagen is attributed to repulsive forces of the surplus negative charges and also to the probable rupture of aldehyde mediated crosslinks.

Amino acids, peptides and related natural products frequently contain two or more functional groups which may react concurrently with α, β—unsaturated compounds. One such compound is acrylonitrile. Acrylonitrile was tested for practical use with wool, both by direct reaction[1, 2] and as a polymerising reagent.[3] Seligsberger and Clayton[4] studied the reaction of acrylonitrile and acrylamide on insoluble collagen. In the present study, the reaction of acrylonitrile with tropocollagen has been investigated under various conditions. Apart from its intrinsic interest, this reaction is of importance to a previous investigation[5] in this laboratory in regard to the ceric ion initiated graft polymerisation of acrylonitrile with collagen.

Experimental

Freeze dried tropocollagen prepared from calf skin was used in this investigation.

Cyanoethylation: Two different conditions were studied. In one experiment, a 0·1% solution of tropocollagen in 0·05N acetic acid was brought to pH 8 with triethylamine and the suspension was treated with aqueous acrylonitrile (ratio of collagen amino group to acrylonitrile 1:160) and kept at 4°C for 3 days with occasional stirring. It was then dialysed exhaustively against 0·05N acetic acid. The viscous solution was centrifuged and any precipitate was discarded. In another set of experiments, freeze-dried tropocollagen was dispersed in cold potassium phosphate-potassium hydroxide buffer of pH 9·7, treated with aqueous acrylonitrile and then dialysed as before. Since glacial acetic acid has often been used as a catalyst in the cyanoethylation of aromatic amines,[6] the possibility of cyanoethylation of collagen in acetic acid was investigated. For this purpose, a solution of tropocollagen in dilute acetic acid was

treated with an aqueous solution of acrylonitrile and after 24 hours, the content of acrylonitrile in the solution was estimated by the method of Beesinq *et al.*[7] using thioglycollic acid, but no decrease in the acrylonitrile content could be detected by comparison with blank tests. Hence, the acid catalysed cyanoethylation of collagen does not appear to occur under these conditions.

Degree of cyanoethylation: This was determined as described by Seligsberger and Clayton.[4]

Viscosity: The specific viscosity of collagen solutions was measured in citrate buffer at pH 3·7 and 20°C in an ubbelhode capillary viscometer from M/s Schott & Genosser, Mainz, Germany.

Denaturation temperature: Using viscosity figures as determined by the foregoing method, the temperature at which the viscosity decrease was half that of the total change from 20 to 40°C, was taken as the denaturation temperature.

Free amino groups: These were determined by the ninhydrin method as described by Cobbet *et al.*[8]

Fibre formation: After trying different conditions and buffers, it was found that fibre formation in the case of cyanoethylated collagen took place more readily in the acid pH than near pH 7. A pH of about 5 was found to be the best. Fibre formation was induced and measured mainly as described by Hafter.[9]

3 ml. of 0·2M acetate buffer with a pH of 5, 0·75 ml. of 2M sodium chloride and 0·2 ml. of 0·5N sodium hydroxide were mixed and made to 4 cc. The solution was cooled and after addition of 2 ml. of collagen solution (0·1% in 0·05N acetic acid) was mixed and heated in a waterbath maintained at 30°C. Increasing turbidity was measured in a Klett summerson photo-electric colorimeter using a green filter. Rigid gelling and maximum turbidity usually appeared within 20 minutes. The samples were all uniformly heated for a period of one hour before inducing dispersion in the cold.

Redispersion of the fibres was studied by placing the opaque gelled system in a waterbath containing ice and measuring the fall in turbidity at definite intervals. At low ionic strength of sodium chloride the fibres formed in the case of cyanoethylated collagen completely dissolved on cooling.

Solubility of cyanoethylated collagen in urea—acetic acid solution

The solubility of the reconstituted cyanoethylated collagen fibrils in urea-acetic acid was determined as described by Bensusan.[10] For this purpose, the fibril formation was carried out as described previously and the collagen was centrifuged. The collagen pellets were placed in individual tubes with 5 ml. of water plus a few drops of toluene and were incubated at laboratory temperature for 24 hours. Afterwards the pellets were placed in a solution of 5 ml. 6M urea which was made 0·1N in acetic acid. After remaining for 24 hours at room temperature, the solution was centrifuged to remove undissolved material and

the supernatant liquid was dialysed 24 hours against running tap water. The amount of solubilised protein was determined by digestion and determination of the hydroxyproline content. It was found that cyanoethylated collagen dissolved completely in urea-acetic acid whereas in the case of untreated collagen the precipitate dissolved only partially.

Dissolution temperature: This was determined as described by Bello and Bello.[11]

Estimation of carbonyl groups: The carbonyl groups in cyanoethylated and untreated tropocollagen were estimated by two methods: (i) by the method of Paz *et al.*[12] using N-methyl benzothiozolone hydrazone and (ii) by the method of Lappin and Clark[13] using 2, 4-dinitrophenyl hydrazine.

Results and discussion

The results obtained by the various methods of analysis cited with untreated and cyanoethylated tropocollagen are shown in Table 1.

The results presented in the table show that no degradation of tropocollagen has taken place during the cyanoethylation process. The free amino groups of tropocollagen have been completely substituted by cyanoethyl groups, free amino groups now being absent. Seligsberger and Clayton[4] were able to introduce about 1·4 m. moles of cyanoethyl groups per g. of insoluble collagen under strongly alkaline conditions and they suggested that ε-amino groups were primarily responsible for the reaction with the reagent. In the present study, however, only about 0·4 m. moles of acrylonitrile could be introduced into tropocollagen. Richm and Scheraga[14] reported that at pH 9·5 all lysine residues of ribonuclease reacted, forming a mixture of mono and bis adducts; however, in the case of β-lactoglobulin only 25% of the ε-amino groups reacted under the same conditions. The absence of free amino groups and the

Table 1

COMPARISON OF TROPOCOLLAGEN AND CYANOETHYLATED TROPOCOLLAGEN°

determination	untreated	cyanoethylated
Reduced viscosity	28.dl/g.	22.dl/g.
Denaturation temperature	38·5°C	35·5°C
Dissolution temperature	50°C	42°C
Free amino groups	0·36*	0·03*
Cyanoethyl groups introduced	—	0·4*
Fibril formation near pH 5 and 30°C	+	+
Fibril formation by dialysis against tap water	+	+
Collagen in 0·05% acetic acid treated with 0·1M acetate buffer to final buffer concentration of 0·025M and pH between 4·6 and 4·8.	Fibres formed	Fibres formed

° Cyanoethylation in triethylamine solution at pH 8.
* Expressed as m.moles per g. of collagen.

amount of cyanoethyl groups introduced into tropocollagen indicate that at pH 8 all the amino groups react to form only mono adducts. Highly alkaline conditions followed by Seligsberger and Clayton[4] are probably necessary for the formation of bis adducts. Under such conditions reaction with guanidino and imidazole groups may also become significant.[15-17] Friedman and Wall[18] have also shown that there is a large drop in the basicities of the amino group on cyanoethylation and that the monocyanoethyl derivative can form bis adducts only under more drastic conditions. Since tropocollagen is prone to degradation under highly alkaline conditions the cyanoethylation in the present study was carried out under mildly alkaline conditions.

From the table, it can be seen that cyanoethylation brings about a decrease in the denaturation temperature by about 3°C. Rauterberg and Kuhn[19] showed that by deamidation and succinylation, the denaturation temperature of collagen was reduced by 3 to 4°C. The studies of Friedman and Wall[18] show that the electrostatic characteristics of proteins will be significantly altered on cyanoethylation of the free amino groups. When collagen is cyanoethylated, the bulky substituents on the amino group combined with an inductive electron withdrawing effect of the cyano group, will favour the monocyanoethyl derivative of the ε-amino group to remain in the uncharged form, and the repulsion forces of the surplus negative charges can lower the stability of the triple helix.[19, 20] Seligsberger and Clayton[4] also found a decrease of 9-14°C in the shrinkage temperature of collagen treated with acrylonitrile.

Besides the decrease in the denaturation temperature, reconstituted cyanoethylated collagen also showed the ability to redissolve completely on cooling whereas the fibrils from untreated collagen treated with buffers alone were only partially soluble. In this respect, cyanoethylated collagen resembles lathyritic collagen. Tanzer[21] reported that collagen treated with thiosemicarbazide behaves like lathyritic collagen with respect to its ability to redissolve on cooling after fibril formation. Lathyrogens such as β-amino propionitrile, induce connective tissue abnormalities which result in an accumulation of soluble collagen and such collagen is found to be deficient in intramolecular crosslinks and incapable of forming stable intermolecular crosslinks *in vitro*. Fressler and Bailey[22] demonstrated that the *in vitro* incubation of β-aminopropionitrile with gelatin derived by mild denaturation of acid soluble rat tail tendon, cleaved β and γ units into α-units by an apparent first order reaction at neutral pH. The inability of lathyritic collagen to produce intermolecular crosslinks has been attributed to its low aldehyde content. Aldehydic compounds are present in collagen and are assumed to participate in its crosslinkage.[23-25] Evidence has been put forward for a rise in extractability of collagen with lathyrism[26] and for the blocking of the maturation process by lathyritic agents.[27] Deshmukh and Nimni[28] recently showed that soluble collagen extracted from insoluble collagen with mercaptoethylamine possessed a high aldehyde content. This solu-

ble collagen, however, aggregated at faster rates than normal collagen and did not dissolve upon cooling. This was attributed to the greater capacity of cysteiamine-soluble collagen to form stable intermolecular crosslinks through the aldehyde groups. The carbonyl content of cyanoethylated tropocollagen was determined by both the methods of Paz *et al.*[12] and also by the method of Lappin and Clark.[13] It was found that cyanoethylated collagen did not give the characteristic blue colour with N-methyl benzothiozolone hydrazone while the 2, 4-dinitrophenyl hydrazine reagent reacted to a lesser extent than untreated tropocollagen.

Cyanoethylation has already been reported[29, 30] for certain aldehydes and ketones. The decreased aldehyde content, lower denaturation temperature and higher solubility of cyanoethylated collagen, therefore, suggest that acrylonitrile may rupture some of the aldehyde mediated crosslinks in collagen and may also combine with the aldehyde groups.

Bello and Bello[11] have shown that collagen fibrils reconstituted from neutral collagen solutions dissolved on heating the fibrillar clot to 50°C. When the collagen fibres were covalently crosslinked as in the case of prolonged incubation at 37°C the collagen fibres showed resistance to dissolving by heat. In the present study, it has been found that cyanoethylated collagen dissolves at a lower temperature than untreated collagen (Table 1). This also suggests that cyanoethylated collagen is less crosslinked than untreated collagen.

Acknowledgment

This research has been financed in part by a grant made by the U.S. Department of Agriculture, Agricultural Research Service, under PL 480.

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